AD_____

Award Number: DAMD17-00-1-0491

TITLE: Pim-1, a Potential Participant in Breast Cancer

PRINCIPAL INVESTIGATOR: Maria Meyer
Nancy S. Magnuson, Ph.D.

CONTRACTING ORGANIZATION: Washington State University
Pullman, Washington 99164-3140

REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual Summary (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
Pim-1, a Potential Participant in Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0491

6. AUTHOR(S)
Maria Meyer
Nancy S. Magnuson, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Washington State University
Pullman, Washington 99164-3140

E-Mail: mkmeyer@wsu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***
Pim-1, a serine/threonine kinase involved in proliferation, differentiation and survival, is a protooncogene involved in cancers of hematopoietic origin. Once thought only to be expressed in hematopoietic cells, it also is expressed in many cell types including epithelial cells. It is therefore suspected that Pim-1 may also play a role in promoting breast cancer. We are examining how Pim-1 expression is regulated in mammary epithelial cells where a signaling pathway called the JAK2/Stat5a (Janus kinase 2/Signal transducer and activator of transcription 5a) pathway, is activated by hormones such as prolactin. This pathway is suspected to regulate *pim-1* transcription. To date, wild type, constitutively active and dominant negative mutants of Stat5a have been made. Mammary epithelial cells have been treated with prolactin and a dose-dependent response has been observed which correlates to Stat5a phosphorylation state. It appears the three cell lines used, which differ in their progesterone and prolactin receptor levels, do not contain equal basal levels of Pim-1 and do not respond identically to prolactin and progesterone stimulation. It is suspected that the ability of these hormones to regulate expression of Pim-1 in mammary epithelial cells allows cells to survive and accumulate mutations resulting in a cell becoming cancerous.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, Pim-1, protooncogene, prolactin, Stat5a, signal transduction	7
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION:

Pim-1 (**P**roviral **i**nsertion site for **M**oloney Murine Leukemia virus) is a serine/threonine kinase shown to be a protooncogene and is involved with cancers of hematopoietic origin. It has many diverse functions which include roles in cell proliferation, differentiation and survival. Most work to date involving Pim-1 has looked at its expression in lymphoid and myeloid cells, the only cells at first thought to express Pim-1. Yet, Pim-1 has recently been found to also be expressed in other cells such as epithelial cells. Therefore it is our hypothesis that Pim-1 is connected with breast cancer, an idea novel to the field of Pim-1 studies and breast cancer research. It is the purpose of this work to examine the possibility that Pim-1 expression in the breast may be regulated by hormones important to the development and function of the breast and that it is this expression, in conjunction with interactions with other proteins, which increases the ability of a cell to survive. Specifically it is proposed that prolactin stimulation activates the JAK2/Stat5a (Janus kinase 2/Signal transducer and activator of transcription 5a) pathway, an occurrence already proven by others, which in turn activates transcription of *pim-1* leading to an increase in Pim-1 protein expression. It is proposed that it is in this manner that Pim-1 is expressed in human mammary epithelial cells.

BODY:

The accomplishments for this period of research in regards to Task 1 include the successful construction of the dominant negative mutant for Stat5a. This construct expresses a truncated Stat5a protein at amino acid 750 and has a Flag tag on the C-terminal end of the protein. Its expression in transfected mammary epithelial cells was confirmed by Western blot with anti-Flag antibody. The wild type, constitutively active mutant (both constructed during the first year) and the dominant negative mutant for Stat5a were all transfected into MCF-7 human mammary epithelial cells. No significant effect on Pim-1 expression could be determined by Western blot. Stat5a is activated by phosphorylation so it is most probable that when these experiments were performed it was overlooked that a signal is needed to phosphorylate the proteins before an effect of their expression can be seen. Upon further analysis of the constitutively active mutant Stat5a protein, it appears that this protein still requires an external signal to become activated and it only acts constitutively once it is phosphorylated. This phosphorylated state is retained for an extended period of time compared to the wild type Stat5a protein causing its constitutive phenotype. Treatment with prolactin should cause this phosphorylation to occur and this treatment is planned for future work.

Another possibility is that an increase in transcription is occurring without a subsequent increase in translation. *pim-1* is a gene regulated at both transcriptional and translational levels. To determine whether these Stat5a constructs were having an effect on the amount of *pim-1* mRNA being expressed and that an effect on protein levels was not seen due to translational controls, RT-PCR (reverse transcription-polymerase chain reaction) was performed a number of times on RNA isolated from cells transfected with the Stat5a constructs. These results were inconclusive due to the need of optimization of

annealing temperatures and it was determined due to this difficulty and the inability of RT-PCR to give solid, quantitative numbers as to the amount of RNA present, a Northern blot will be performed. This is a better procedure to use in obtaining an answer as to whether translational control is causing the inability to see an effect on Pim-1 expression when the Stat5a constructs are introduced.

Another part of Task 1 was to examine the dose-dependent effects of prolactin treatment using a beta-casein promoter reporter as a way to measure activation of Stat5a. Beta-casein is a gene which is regulated by prolactin through activation of the JAK2/Stat5a pathway. Therefore the rat and human beta-casein promoters were obtained as a gift from Dr. Wolfgang Doppler (University of Innsbruck, Austria). To make the promoter-reporter construct, the cytomegalovirus promoter was first removed from pEGFP-N1 (a Green fluorescent protein (GFP) expression vector from Clontech) then the beta-casein promoter was subcloned into the now promoterless GFP expression vector creating the plasmid pbetac(-344/-1)GFP. This plasmid can thereby act as a transcription reporter system for Stat5a activation. GFP will only be expressed when the beta-casein promoter is used and therefore, since beta-casein is a gene regulated by prolactin through Stat5a, it can be determined whether Stat5a is activated in cells by transfecting cells with this plasmid and measuring GFP expression by either fluorescence microscopy or by FACS (fluorescence activated cell sorter) analysis. This plasmid (pbetac(-344/-1)GFP) was co-transfected with the Stat5a constructs into MDA-MB-231 mammary epithelial cells and it appeared, by fluorescence microscopy, that the Stat5a constructs affected the expression of GFP but since this method relied on the subjective eye of the researcher to determine the number of cells fluorescing, a more definitive quantitative answer was attempted to be obtained by repeating this using FACS analysis. FACS analysis allows the counting of single cells expressing GFP and gives numbers of cells expressing GFP relative to those not. It was determined at this time that the transfection efficiency of control cells was too low to make any significant conclusions. Also since the time that these experiments were performed, a luciferase plasmid has been obtained to act as a control for transfection efficiency since this was a factor not initially considered in these experiments. Therefore these experiments will be repeated after optimization of transfection (a procedure already performed) and use of the luciferase plasmid will be included to normalize the transfection efficiency between the different transfection samples.

This past year two more human mammary epithelial cell lines have been examined as to effects hormones (prolactin and progesterone) have on Pim-1 expression. In addition to MCF-7 cells, MDA-MB-231 cells and T47D-co cells have also been used. MDA-MB-231 cells have a low prolactin and progesterone receptor level while T47D-co cells constitutively express progesterone receptor without the need for estrogen pretreatment. The expression of hormone receptors can actually be regulated by each other and therefore this cell line, T47D-co, is a nice line to work with since it takes out one of the variables of regulation. The preliminary findings suggest that these cells will provide the nicest data in regards to dissecting the prolactin-induced regulation of Pim-1. Therefore it is felt that these are good cell lines to work with since they differ in their receptor profiles. The significant findings of hormone treatments with all three of these

cell lines are discussed here. T47D-co cells were cultured in 10% gelding serum for one day then stimulated with ranging concentrations of prolactin and samples taken at time 0, 10 minutes, 1 hour and 2 hour. It was found that 150 ng/ml prolactin caused in increase in Pim-1 expression at one hour which fell back down at two hours to levels equal to that at 10 minutes. 1 μg/ml prolactin caused a large increase in Pim-1 expression at 10 minutes which rapidly decreased at hour one and even more so at hour two. It is not yet known but is suspected that rapid protein degradation is causing this rapid decrease in Pim-1 levels. 5 ng/ml and 50 ng/ml were also used to examine their effects on Pim-1 expression and the amount of increase was found to not be as great at these concentrations showing that the increase in expression is concentration dependent.

Research performed related to the parts in Task 2 is as follows. Previous work with T47D-co cells has shown that treatment of these cells with progesterone causes an increase in Stat5a levels (Richer et al., 1998). To determine if this could also be shown in our lab, as well as to show that prolactin stimulation was in fact causing an effect on Stat5a phosphorylation in our own hands, T47D-co cells cultured for one day in 10% gelding serum were either pretreated with progesterone for 48 hours or not, and then treated with or without prolactin for 10 minutes. Cell lysates were then made and immunoprecipitations performed using anti-Stat5a antibody. The isolated protein-antibody complex was isolated using Protein G beads and it along with cell lysates were run on a gel to perform a Western blot. The blot containing the immunoprecipitations was probed with anti-phosphotyrosine and the cell lysates portion of the blot was probed with anti-Stat5a. It was observed, as expected, that the pretreatment of cells with progesterone increased the level of Stat5a and that treatment of the cells with prolactin caused phosphorylation of Stat5a. Only one concentration of progesterone has been used at this time so dose-dependent effects on the level of Stat5a protein in our lab can not be concluded at this time though this is an effect shown by others already. The highest level of phosphorylation was seen with 1 μg/ml prolactin with less being seen with 150 ng/ml. This again shows the dose dependent effects of treatment with prolactin. This is an important step in closing the loop as to what causes an increase in Pim-1 expression when stimulated with prolactin. The next step will involve transfection of the Stat5a constructs followed by the same procedure described above and examination of Pim-1 levels will follow.

To investigate the effect progesterone has on Pim-1 expression, cells were treated for 24 hours with 30 nM progesterone. No effect on Pim-1 expression was observed. MDA-MB-231 cells serum starved for three days then stimulated with 1 nM – 10 μM progesterone for two hours showed no effect on Pim-1 expression. MCF-7 cells were pretreated with 30 nM progesterone for 48 hours then restimulated with 30 nM progesterone and samples were taken at 10 minutes and two hours. An interesting finding which needs further examination is an upper band which appears to be regulated by this restimulation. This band falls about 38-40 kilodaltons (KD) on Western blot and is present at time 0 and 10 minutes but decreases significantly at hour 2. The other two cell lines do not show this effect. Pim-1 is a protein 33-34 KD in size so it is suspected, due to this finding and other findings in our lab, that this upper band may possibly be a modified form of Pim-1. Another interesting finding is that all three human mammary

cell lines examined (MDA-MB-231, MCF-7 and T47D-co) have different profiles when blotted for Pim-1. It appears that the cell line MDA-MB-231 has the highest level of basal Pim-1 expression and T47D-co has the lowest level of expression.

KEY RESEARCH ACCOMPLISHMENTS:

- Expression of the human dominant negative mutant Stat5a flag-tagged protein in transfected MCF-7 cells
- Observation of differential regulation of Pim-1 protein by prolactin and progesterone dependent on dose and cell line
- Observation of a correlated increase in Pim-1 protein expression and Stat5a phosphorylation when T47D-co cells are stimulated with prolactin and/or progesterone

REPORTABLE OUTCOMES: none

CONCLUSIONS:

Although many solid research accomplishments are not yet able to be made, it is felt that the system is now up and running in such a way that this next year will be quite successful in completely addressing all proposed research. In looking back, many experiments have been started and remain to be completed with solid controls in order to definitively come to a significant finding. All techniques (i.e. Northern blots, FACS analysis, transfections, etc.) which should allow for this to be done have been learned by the researcher in the past two years and all of the tools (i.e. plasmids) have been made, a process which took a large amount of time to complete. It is for these reasons that this next year is begun with great confidence that successful data collecting will occur. The fact that it has been observed that prolactin stimulation causes an increase in Pim-1 expression and that effects on Stat5a levels and phosphorylation can be observed by the researcher gives strong footing to being able to complete research which will allow for important reportable outcomes to be made.

REFERENCE:

Richer, J. K., Lange, C. A., Manning, N. G., Owen G., Powell, R., and Horwitz, K. B. (1998) *J. Biol. Chem.* **273**, 31317-31326.